Twice vitrification-warming procedures has no effect on frozen-thawed embryo transfer outcomes

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Research

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Abstract

Background

To evaluate the frozen-thawed embryo transfer (FET) outcomes of repeated cryopreservation by vitrification of blastocysts derived from vitrified-warmed day3 embryos in patients who experienced implantation failure previously.

Methods

We retrospect the files of patients who underwent single frozen-thawed blastocyst transfer cycles in our reproductive medical center from January 2013 to December 2019. 127 patients transfer of vitrified-warmed blastocysts derived from vitrified-warmed day3 embryos were defined as twice-cryopreserved group. 1567 patients who transfer blastocysts that had experienced once vitrified-warmed were used as once-cryopreserved group. None of them was pregnant at the previous FET. The outcomes were compared between two groups after a 1:1 propensity score matching (PSM).

Results

The clinical pregnancy rate was 52.76%, live birth rate was 43.31% in twice-cryopreserved group. After PSM,108 pairs of patients were generated for comparison. The clinical pregnancy rate, live birth rate or miscarriage rate was not significantly different between two groups. Logistic regression analysis indicated that double vitrification-warming procedures did not affect FET outcomes in terms of clinical pregnancy rate (OR 0.83, 95%CI 0.47-1.42), live birth rate (OR 0.93, 95%CI 0.54-1.59), miscarriage rate (OR 0.72 95%CI 0.28-1.85). Furthermore, the pregnancy complications rate, gestational age or neonatal abnormalities rate between two groups was also comparable, while twice vitrification-warming procedures might increase the macrosomia rate (19.6% vs. 6.3%, P = 0.05).

Conclusion

Transfer of double vitrified-warmed embryo at cleavage stage and subsequent blastocyst stage did not affect live birth rate and neonatal abnormalities rate, but there was a tendency to increase macrosomia rate, which needs further investigation.

Background

It has been decades since the first IVF baby born, and then assisted reproduction has acquired significant advances,the number of available embryos in each IVF cycle has also increased, resulting in a slew of surplus embryo cryopreservation. Cryopreservation of embryos will increase the cumulative pregnancy rate for a single cycle of ovarian stimulation, the clinical pregnancy rate and live birth rate was similar with fresh embryo transfer, while frozen-thawed embryo transfer (FET) decreases the risk of ovarian hyperstimulation syndrome (OHSS), provides optimal endometrial preparations in case of premature progesterone rises, adenomyosis or

hydrosalpinx [1–3]. Embryo cryopreservation has become a widespread reliable procedure in assisted reproductive technologies [4]. However, cryopreservation procedure may be have negative effects on both mothers and babies when compared with fresh embryo transfer such as higher rate of hypertensive disorders of pregnancy, large for gestational age (LGA) and higher birthweight [5–7].

Single embryo transfer was global recommended to reduce multiple pregnancies[8]. In case of multiple embryos were frozen in one cryo-straw previously, these vitrified embryos will be warmed together, when only one embryo were transferred, the other warmed embryos have to be vitrified again, so these embryos would undergo twice vitrification-warming procedures. Some studies have focused on whether repeated cryopreservation process affect the pregnancy and perinatal outcomes. One study showed that twice-frozen-thawed embryos have a lower post-thaw survival rate but equivalent pregnancy outcomes to once-frozen embryos[9]. Others found repeated cryopreservation process impairs embryo implantation potential, with lower birth rate and higher miscarriage rate[10, 11]. However, the patients underwent repeated cryopreserved embryo transfer may be always experienced previous embryo transfer failure, so the control group should be consistent with this, which may the sample selection bias in these studies. And the most common situation in our center was that two embryos were frozen in one cryo-straw at cleavage stage, after single embryo transfer, the surplus thawed embryos will culture to blastocyst stage and vitrified again, so these embryos underwent twice vitrification-warming procedures at cleavage stage and subsequent blastocyst stage, which was different from some other studies when the repeated cryopreservation process was performed at the same stage.

In this retrospective study, we investigated the effectiveness and safety of twice vitrification-warming procedures at cleavage stage and subsequent blastocyst stage in in patients who experienced previous failure. The pregnancy and perinatal outcomes were compared between the twice-cryopreserved group and the once-cryopreserved group.

Patients And Methods

Patients

The medical files of all frozen-thawed blastocyst cycles from January 2013 to December 2019 at Reproductive Medicine Center of Nanjing Drum Tower Hospital were retrospectively reviewed. We established inclusion and exclusion criteria. Inclusion criteria：(1) single blastocyst transfer, (2) the patient had experienced at least one frozen-thawed cycle and had not received a live birth in the precede cycle. Exclusion criteria：(1) patients received multiple births, (2) endometriosis, (3) preimplantation genetic testing cycles, (4) donor cycles, (5) history of recurrent pregnancy loss, and (6) uterine pathology. Totally 1694 women patients were included in this study. They were divided into two groups based on times of embryos had been cryopreserved：once-cryopreserved group（n=1567） and twice-cryopreserved group（n=127）. Among twice-cryopreserved group, 127 FET cycles were those patients in whom two embryos were frozen by vitrification in one cryo-straw at cleavage stage, after single embryo transfer, the surplus embryo will culture to blastocyst stage in vitro, then refrozen by vitrification, then transferred after rethawing.

IVF/ICSI procedure and embryos culture

Depending on the patients' age, ovarian reserve function, and ovarian responses in the previous ovulation cycle, gonadotrophin-releasing hormone agonist or antagonist protocols were used. When two to three dominant follicles reached a diameter of 18 mm, human chorionic gonadotropin (HCG) was injected. The oocytes were retrieved under transvaginal ultrasound guidance 36-38 hours after HCG administration. Retrieved oocytes were then fertilized in conventional IVF or intracytoplasmic sperm injection (ICSI). Two pronuclei and a second polar body were examined for normal fertilization at 16–18 h post-insemination. Embryos were cultured in G1/G2 sequential media (Vitrolife, Goteborg, Sweden) at 37°C in a 6% CO2, 5% O2 and 89% N2 high-humidity incubator. Blastocyst morphological evaluation was based on the Gardner scoring system [12].

Vitrification cryopreservation and thawing procedures

The vitrification cryopreservation and thawing procedures was performed in embryos，vitrification kit was produced and available at Kitazato Co., Fujinomiya, Japan. Before vitrification, the blastocyst was artificially shrunken by laser drilling.

Cleavage embryos or shrunken blastocysts were equilibrated in equilibration solution for 5–8 min at room temperature, then placed into vitrification solution, 1 minute later the embryos were loaded onto the cryotop strip and plunged into liquid nitrogen immediately. For warming, the cryotop strip was removed from the liquid nitrogen and immediately transferred to thawing solution for 1 minute at 37◦C, dilution solution for 3 minutes, and then washed twice in washing solution 1 and washing solution 2 for 5 min each. After warming, embryos were cultured in embryo culture medium at 37°C in 6% CO2 for 2–4 h until transfer.

Outcome Measures

Baseline demographics for each patient included age, BMI, duration of infertility, parity, type of infertility and cause of infertility, insemination methods (IVF or ICSI), endometrial thickness，and endometrium preparation protocol for FET.

Serum HCG was measured after embryo transfer 12–14 days. Clinical pregnancy was defined as the presence of gestational sacs observed on an ultrasound scan at least 5 weeks after the embryo transfer. The fetal loss before 28 weeks was defined as miscarriage. The embryo was implanted outside the uterine cavity called ectopic pregnancy. Such as hypertensive disorders of pregnancy, gestational diabetes mellitus, intrahepatic cholestasis during pregnancy and placenta previa were included in pregnancy complications. Any singleton birth ≥28 weeks of gestation was considered a live singleton birth. The neonatal outcomes evaluated were gestational age, preterm birth (delivery between 28 and 37 weeks), low birthweight (birthweight <2500 g)， macrosomia (birthweight≥4000 g)，gender and neonatal abnormality.

Statistical analysis

Means ± SD for continuous variables，percentages for categorical variables were calculated. The data were analyzed using the chi-squared test or Student’s t-test. P- values <0.05 was considered statistically significant. 1:1 Propensity score matching was used in data analysis to eliminate the influence of baseline characteristics on outcomes. The differential variables include female age at retrieval, average embryo transfer cycles, parity, basal FSH, type of infertility and cause of infertility. To analyze the associations between the cryopreserved times and pregnancy outcomes, logistic regression models were conducted for each outcome indicator using

the after-matching data, and odds ratio (OR) and its 95% CI before and after adjusting for confounders were calculated. Statistical analysis was performed using SPSS statistical package version 26.0.

Results

Clinical characteristics and outcomes of the twice-cryopreserved grou p

Patients' overall baseline clinical demographics of the twice-cryopreserved group were displayed in Table 1. The female age at retrieval or embryo transfer were 29.61±3.96 and 34.28±4.12 years. The average BMI and basal FSH of the study population were 22.33±2.89 kg/m^2 and 6.7±1.77 mIU/mL, respectively. In the FET cycles, the hormone-replacement therapy (HRT) predominated in endometrium preparation (104 cycles). In the 127 blastocysts, ninety-one were day 5 blastocysts, while the remaining 36 were day 6 blastocysts.

Table 1
Overall demographics,baseline IVF characteristics of the twice-cryopreserved group (n=127).

Demographics	
Female age at retrieval ,years	29.61±3.96
Female age at embryo transfer,years	34.28±4.12
Parity	0.78±0.47
Body mass index,kg/m^2	22.33±2.89
Basal FSH, mIU/mL	6.70±1.77
Duration of infertility ,years	4.09±2.65
Endometrial thickness, mm	9.32±1.68
Type of Infertility, n	
Primary	71
Secondary	56
Cause of Infertility, n	
Ovulation disorder	6
Tubal factor	78
Male factors	11
Other factors	32
IVF characteristics	
Insemination method, n	
IVF	96
ICSI	31
Average number of embryo transfer cycle	3.65±1.12
Endometrial thickness, mm	9.32±1.68
Endometrial preparation protocol, n	
HRT	104
Ovulation induction	13
Modified Natural Cycle	10
Day of embryo cryopreservation, n	
D5	91

Demographics	
D6	36
Embryo level, n104	
High quality embryos	76
Good quality embryos	51

Clinical outcomes and perinatal outcomes of the study population were listed in Table 2. The clinical pregnancy rate and live birth rate were 52.76% and 43.31%. In all clinical pregnant people,14.93% patients occurred miscarriage before 12 weeks, 11 pregnant women had pregnancy complications and no ectopic pregnancy occurred. According to the follow-up results, 55 live singleton deliveries were obtained, including 28 male babies and 23 female babies. The average gestational age was 37.65±2.21weeks, and the average birthweight was 3412.60±666.37g. The preterm birth in singletons was 16.36%, and rate of low birthweight and macrosomia were 7.27% and 16.36%, respectively. There was no neonatal disease and malformation occurred.

Table 2
Clinical outcomes and perinatal outcomes of twice-cryopreserved embryos transferred (n=127).

Clinical Outcomes	
Biochemical pregnancy rate (%)	72/127 (56.69)
Clinical pregnancy rate (%)	67/127 (52.76)
Miscarriage rate (%)	11/67(16.42)
Early (<12 weeks)	10/67 (14.93)
Late (12–28 weeks)	1/67 (1.49)
Ectopic pregnancy	0/67 (0)
Pregnancy complication (%)	11/67 (16.42)
Live birth rate (%)	55/127 (43.31)
Perinatal outcomes	
Singleton delivery	55
Gestational age ,weeks	37.65±2.21
Preterm birth rate (<37 weeks) (%)	9/55(16.36)
Full-term birth rate (37-42 weeks) (%)	46/55(83.63)
Gender	
Boys	28
Girls	27
Neonatal abnormalities (%)	0/55(0)
Birthweight ,g	3412.60±666.37
Low birthweight (<2500 g) (%)	4/55(7.27)
Macrosomia (≥4000g) (%)	9/55(16.36)

The propensity score matching of basic characteristics between once-cryopreserved group and twice-cryopreserved group

Among a total of 1694 embryos, 127 were re-thawed and underwent transfer (twice-cryopreserved group), 1567 were underwent a single thawed embryos transfer (once-cryopreserved group). The comparison of clinical characteristics between the two groups were listed in Table 3. The female patients were younger (29.61±3.96 vs. 31.42±4.96, p<0.001), basal FSH was lower (6.70±1.77 vs. 7.53±3.19, p<0.001) in twice-cryopreserved group. More embryo transfer cycles were performed in the twice-cryopreserved group (mean cycles:3.65) than in once-cryopreserved group (mean cycles: 2.94) (P < 0.001) and the parity was more in the twice-cryopreserved

group (0.78±0.47 vs. 0.32±0.49, P < 0.001). The distribution of infertility type, cause of infertility was different between the two groups.

Table 3 The propensity score matching of basic characteristics between once-cryopreserved group and twice-cryopreserved group.

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In order to eliminate the influence of baseline characteristics on outcomes, a 1:1 PSM was performed between the two groups. Finally, a total of 108 pairs were matched by their propensity score. Before propensity score matching, two groups had significantly different maternal and IVF characteristics but the propensity score matching balanced these characteristics between the cohorts (Table 3), suggesting the matched cohorts had highly similar baseline characteristics.

Pregnancy outcomes between the once-cryopreserved group and twice-cryopreserved group after matching

The clinical outcomes were summarized in Table 4. The clinical pregnancy rate (51.85% vs. 57.41%, p=0.41), live birth rate (44.44% vs. 42.59%, p=0.78) and miscarriage rate (16.07% vs. 20.97%, p=1.00) were not significantly different between the two groups. No late miscarriage and ectopic pregnancy occurred in both groups. There were 5 patients with pregnancy complications in once-cryopreserved group, and 7 patients with pregnancy complications in twice-cryopreserved group. There was no statistical difference in the incidence of pregnancy complications (12.50% vs. 8.06%) between the two groups.

	Before matching			After matching		
	Once-cryopreserved group	Twice-cryopreserved group	P-value	Once-cryopreserved group	Twice-cryopreserved group	P-value
No. of cycles	1567	127	-	108	108	-
Female age at retrieval，years	31.42±4.96	29.61±3.96	<0.001	29.94±4.86	29.88±4.05	0.93
Average embryo transfer cycles	2.94±1.08	3.65±1.12	<0.001	3.28±1.35	3.58±1.06	0.07
Parity	0.32±0.49	0.78±0.47	<0.001	0.69±0.54	0.75±0.50	0.36
Body mass index, kg/m^2	22.64±3.27	22.33±2.89	0.25	22.43±2.90	22.39±3.05	0.93
Basal FSH ,mIU/mL	7.53±3.19	6.70±1.77	<0.001	7.00±2.31	6.74±1.78	0.34
Duration of infertility, years	3.95±3.16	4.09±2.65	0.61	4.13±2.97	4.28±2.70	0.69
Endometrial thickness,mm	9.45±1.69	9.32±1.68	0.38	9.26±1.31	9.32±1.75	0.76
Insemination method			0.90			0.54
IVF	1177	96		76	80	
ICSI	390	31		32	28	
Type of Infertility			0.02			0.89
Primary	711	71		57	58	
Secondary	856	56		51	50	
Day of embryo cryopreservation			0.30			0.42
D5	1053	91		86	81	
D6	514	36		22	27	
Endometrial preparation protocol			0.16			0.81
HRT	1269	104		86	88	
Ovulation induction	148	13		11	11	
Natural Cycle (NC)	150	10		11	9	
Cause of			<0.001			0.25

Infertility					
Ovulation disorder	73	6	1	4	
Tubal factor	648	78	58	65	
Male factors	166	11	16	11	
Other factors	680	32	33	28	
Embryo quality		0.25			0.34
High quality embryos	947	76	56	64	
Good quality embryos	620	51	52	44	

Table 4 The clinical outcomes between once-cryopreserved group and twice-cryopreserved group after propensity score matching.

	Once-cryopreserved group	Twice-cryopreserved group	P-value
No. of cycles	108	108	-
Biochemical pregnancy rate (%)	70/108 (64.81)	61/108 (56.48)	0.21
Clinical pregnancy (%)	62/108 (57.41)	56/108 (51.85)	0.41
Miscarriage (%)	13/62 (20.97)	9/56 (16.07)	1.00
Early (<12 weeks)	13/62 (20.97%)	9/56 (16.07)	
Late (12–28 weeks)	0/62 (0.0)	0/56 (0.0)	
Ectopic pregnancy (%)	0/62 (0.0)	0/56 (0.0)	1.00
Live birth rate (%)	48/108 (44.44)	46/108 (42.59)	0.78
Pregnancy complication (%)	5/62 8.06	7/56 12.50	0.43

Moreover, we used regression model to adjust factors that might affect the results according to female retrieval age, BMI, FSH, embryo transfer cycles, embryo quality. The adjusted outcome showed that the clinical pregnancy rate (AOR 0.83, 95%CI 0.47, 1.42), live birth rate (AOR 0.93, 95%CI 0.54, 1.81) or early miscarriage rate (AOR 0.72, 95%CI 0.28, 1.85) was no statistical difference (Table 5).

Table 5
Adjusted Clinical pregnancy rate/Live birth rate/Early miscarriage after propensity score matching.

Embryo cryopreservation times	Clinical pregnancy rate (%)			Live birth rate (%)			Early spontaneous abortion(%)		
	%	AOR	95%CI	%	AOR	95%CI	%	AOR	95%CI
Once	57.41	1	Reference	44.44	1	Reference	20.97	1	Reference
Twice	51.85	0.83	0.47-1.42	42.59	0.93	0.54-1.59	16.07	0.72	0.28-1.85

We adjusted the female retrieval age, BMI, FSH, embryo transfer cycles, parity, the type of infertility and the quality of embryos. AOR is the adjusted odds ratio in logistic regression analysis, representing the risk ratio of clinical pregnancy/live birth/early miscarriage in the twice-cryopreserved group versus once-cryopreserved group. AOR greater than 1 represents increased risk, and AOR less than 1 represents reduced risk. 95% CI is the 95% confidence interval of AOR.

In addition, we conducted a stratified analysis based on the day of embryo cryopreservation, embryo quality and previous labor history, the difference in live birth rates between the two groups is shown in Table 6. The results showed that transferring D5 or D6 blastocyst,delivery history or not, the live birth rate had no significant difference between the two groups (P>0.05). In both groups, high-quality blastocysts for transferring resulted in a higher live birth rate than good-quality blastocysts (P<0.01). However, there was also no significant difference in the live birth rate on transferring the same level embryos between the two groups.

Table 6
The live birth rate analyzed by stratification between once-cryopreserved group and twice-cryopreserved group after propensity score matching.

	Once-cryopreserved group		Twice-cryopreserved group		P-value
	Live birth rate (%)	P-value	Live birth rate (%)	P-value	
Day of embryo cryopreservation		0.87		0.26	
D5	39/87(44.83)		37/81(45.68)		0.91
D6	9/21(42.86)		9/27(33.33)		0.50
Embryo quality		0.08		<0.01	
High quality embryos	30/56(53.57)		35/64(54.69)		0.90
Good quality embryos	18/52(34.6)		11/44(25)		0.28
Parity		0.11		0.47	
No,Primipara	13/38(34.21)		14/29(48.28)		0.24
Yes,Multipara	35/70(50)		32/79(40.51)		0.24

Neonatal characteristics of live-born singletons between the once-cryopreserved group and twice-cryopreserved group after matching

Of the 216 embryo transfer cycles, there were a total of 94 delivered singletons, including 46 derived from the twice-cryopreserved group and 48 from the once-cryopreserved group. There were no significant differences in the mean gestational age (37.85±2.01 vs. 37.65±1.92, P=0.62), gender ratio (girls/boys,23/23 vs. 25/23, P=0.84), mean birthweight (3497.02±642.01 vs. 3371.98±522.86, P=0.30), macrosomia rate (19.56% vs. 6.35%, P=0.05) and preterm birth rate (13.04% vs. 12.5%, P=0.94) between the two groups (Table 7). A singleton baby born in the once-cryopreserved group had angioma defect and the rate of congenital birth defects was 1/48 in the once-cryopreserved group. While no newborn anomalies were occurred in the twice-cryopreserved group.

Table 7
Neonatal characteristics of live-born singletons between once-cryopreserved group and twice-cryopreserved group after propensity score matching.

	Once-cryopreserved group	Twice-cryopreserved group	P-value
No. of cycles	108	108	
Live-born singletons	48	46	
Gestational age, weeks	37.65±1.92	37.85±2.01	0.62
Preterm birth (<37 weeks) (%)	6/48 (12.5)	6/46 (13.04)	0.94
Term birth (≥37 weeks) (%)	42/48 (87.5)	40/46 (86.96)	0.94
Neonatal abnormalities (%)	1/48 (2.08)	0/46 (0.0)	1.00
Birthweight, g	3371.98±522.86	3497.02±642.01	0.30
Low birthweight (<2500 g) (%)	0/48(0.0)	0/46 (0.0)	1.00
Macrosomia (≥4000g) (%)	3/48 (6.35)	9/46 (19.56)	0.05
Gender			
Boys	23	23	
Gestational age, weeks	37.83±1.34	37.70±1.96	0.79
Birthweight, g	3503.48±384.77	3598.26±723.09	0.58
Low birthweight (<2500 g) (%)	0/23(0.0)	0/23 (0.0)	1.00
Macrosomia (≥4000g) (%)	2/23 (8.69)	6/23 (26.09)	0.24
Girls	25	23	
Gestational age, weeks	37.48±2.35	38.00±2.09	0.42
Birthweight, g	3251.00±606.50	3395.78±546.63	0.39
Low birthweight (<2500 g) (%)	0/25 (0.0)	0/23 (0.0)	1.00
Macrosomia (≥4000g) (%)	1/25 (4.0)	3/23 (13.04)	0.26

In order to eliminate the possible effect of infant gender on birthweight, we compared the differences in offspring birth weight and mean gestational weeks between the once-cryopreserved group and the twice-cryopreserved group in boys and girls, respectively (Table 7). The results shown that both boys and girls, there was no significant difference in gestational week and the birth weight between the two groups. Whether boys or girls, the macrosomia rate in the twice-cryopreserved group was slightly higher than that in the once-cryopreserved group, but it did not reach statistical difference (P>0.05).

Discussion

Embryo cryopreservation is a crucial part of assisted reproductive technology, and the proportion of FET had grown remarkably. In order to reduce the risk of multiple pregnancies and pregnancy complications, single embryo transfer is preferred in the case of suitable patient conditions and embryo quality. Studies have found that compared with embryos in cleavage stage, single blastocyst transfer significantly improves clinical pregnancy rate and implantation rate [13–16]. As multiple cleavage stage embryos might be frozen in one cryo-straw previously, after single embryo transfer, the surplus thawed embryos will be cultured to blastocyst stage and vitrified again, if this time of embryo transfer failed, these patients might choose to transfer these embryos which underwent twice vitrification-warming procedures. In this study, we found that transfer of twice vitrified-warmed embryo at cleavage stage and subsequent blastocyst stage did not affect pregnancy and perinatal outcomes.

There were several case reports demonstrating healthy live birth after a frozen embryo transfer with embryo that were frozen and thawed twice [17–21]. And some early retrospective studies have also shown that the embryos re-cryopreservation does not affect clinical pregnancy rates [9, 22–24]. However, other recent studies have shown that transfer of twice frozen-thawed embryos increase the rate of miscarriage and decreases the clinical pregnancy rate and live birth rate [10, 11, 25]. The discrepancies in the results of these studies may be due to the different methods used to freeze embryos,embryos frozen at different stages and mismatched baseline of clinical characteristics. Furthermore, these studies may exist the sample selection biases, as the patients underwent repeated cryopresered embryo transfer may always experience previous embryo transfer failure, so the control group should be consistent with this. So, the control group in our study were also experienced previous embryo transfer failure. Furthermore, in order to eliminate the influence of clinical baseline characteristics on results, a propensity score and logistic regression was used to control for potential confounding differences in our study.

After PSM, the clinical pregnancy rate, miscarriage rate, live birth rate or the incidence of pregnancy complications had no difference between the once-cryopreserved group and the twice-cryopreserved group. There was a difference in clinical pregnancy and live birth rates between blastocysts developing on Day 5 (D5) and Day 6 (D6),the clinical pregnancy rate and live birth rate were significantly higher following D5 compared to D6 blastocysts [26, 27]. In addition，the quality of embryos plays a crucial role in embryo implantation and live birth rate[28, 29]. In the present study, we stratified the live birth rates based on the day of embryo cryopreservation, embryo quality between once-cryopreserved group and twice-cryopreserved group and found no significant difference. Logistic regression after adjusting for relevant confounding factors showed that the number of vitrification-warming procedure was not related to the clinical pregnancy rate, live birth rate or early miscarriage rate, which was not consist with other studies. Zheng et al. (2017) reported the miscarriage rate of the twice-cryopreserved group was higher and the live birth rate was lower than control group, which may due to two cryopreservation methods were used in their study, slow freezing of cleavage embryos followed by vitrification of blastocysts[10]. In our study, we only used one cryopreservation method, vitrification. Compared with slow-freezing method, embryo vitrification is an ultra-rapid cryopreservation method which prevent ice formation, reduce embryo cryoinjury, have higher embryo survival rate and live birth rate [30, 31]. One other study as well indicated that the embryo implantation rate, clinical pregnancy rate and live birth rate in the re-cryopreservation group were significantly lower, and the miscarriage rate also slightly increased[11]. In their study, embryos underwent twice cryopreservation in the blastocyst stage, in addition embryo selection was restrictive compared with once cryopreservation group. Moreover, the blastocyst underwent twice frozen-

thawed, and the times of laser drilling and shrinkage increased, which may affect the implantation ability of embryos. Although current studies have confirmed the safety of laser manipulation in human embryo cryopreservation, however the cumulative effect is unclear[32, 33]. In our study, we selected embryos underwent twice-cryopreservation, once at the cleavage stage and the second time at the blastocyst stage. Blastocyst culture itself was the selection of embryos, only an embryo with developmental potential can form a blastocyst. And the blastocysts underwent the same number of laser drilling as in the one-freeze group.

More and more children are born depending on ART globally, so the safety of the offspring is more concerned. When compared with fresh embryo transfer,FET is associated with higher risk of LGA and macrosomia in singletons[5, 7, 34]. The maternal and fetal health that experiences repeated freezing should be paid more attention to. Our results showed that no significant differences were observed in pregnancy complication rate, gestational age, fetal birthweight, gender, congenital birth defects rate in the singleton delivery between once-cryopreserved and twice-cryopreserved group. However, the macrosomia rate was 19.56% in the twice-cryopreserved group, when it was 6.35% in once-cryopreserved group, whether the time of vitrification-warming procedure is positively associated with the fetal birthweight need further study. Birthweight may be related to gender, so the incidence of macrosomia, gestational age, birthweight was further analyzed for boys or girls separately, and no significant differences was observed may due to few samples.

There are several limitations in our study. First, it was a retrospective study conducted in a single center, a larger prospective cohort study was needed to validate our findings. Second, although we used propensity score matching to eliminate the influence of baseline characteristics on FET outcomes as much as possible, it could not eliminate all the bias caused by confounders between the two groups.

In summary, our results presented the pregnancy outcomes and neonatal safety of human refrozen-thawed embryos. Twice vitrification of human embryos at different developmental stages do not affect clinical outcomes and perinatal outcomes. The re-cryopreservation procedure would be a valuable option to increase the cumulative pregnancy rate while preventing embryo waste under full informed consent. However, the maternal and child safety of re-cryopreservation procedure still needs to be confirmed by a long-term multicenter prospective cohort study.

Abbreviations

FET: frozen-thawed embryo transfer; PSM: propensity score matching; CPR: clinical pregnancy rate; LBR: live birth rate; IVF: in-vitro fertilization; OHSS: ovarian hyperstimulation syndrome; LGA: large for gestational age; BMI: body mass index; FSH: follicle-stimulating hormone; HRT: hormone-replacement therapy; HCG: human chorionic gonadotropin; ICSI: intracytoplasmic sperm injection.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Nanjing Drum Tower Hospital, The Affiliated Hospital to Nanjing University Medical School on 6 May 2021 (reference number 2021-163-01). This study was

conducted at Reproductive Medicine Center of Nanjing Drum Tower Hospital. All patients signed informed consents regarding ART.

Consent for publication

Not applicable.

Availability of data and materials

Please contact author for data requests.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

JZ contributed to the study design. MD, JX collected data and conducted analysis. YY contributed analysis tools. SW helped to perform the laboratory operations. XS was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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